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Monoclonal Antibody-Based Competitive Enzyme-Linked Immunosorbent Assay for Detecting and Quantifying West Nile Virus-Neutralizing Antibodies in Horse Sera[▽]

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A rapid immunoassay for detecting and quantifying West Nile virus (WNV)-neutralizing antibodies in sera was developed as an alternative to the plaque reduction neutralization test (PRNT), the gold standard test for WNV. The assay is a competitive, enzyme-linked immunosorbent assay using neutralizing monoclonal antibody 5E8 (NT-ELISA). A cutoff percent inhibition (PI) value of 35% (mean PI plus 3 standard deviations), with a specificity of 99%, was established based on analysis of 246 serum samples from horses free of WNV. The NT-ELISA detected neutralizing antibodies in all sera collected 7 or 14 days postinoculation from mice ($n = 11$) infected with lineage I (strain NY385-99) or II (strain B956) WNV. When sera from WNV-vaccinated horses ($n = 212$) were tested by NT-ELISA and PRNT, the NT-ELISA gave a positive result for 96.1% (173/180) of the PRNT-positive sera and 3.1% (1/32) of the PRNT-negative sera. Discrepancies between the two tests were observed mainly with sera with low $PRNT_{90}$ titers (expressed as the reciprocal of the highest dilution yielding ≥90% reduction in the number of plaques) for WNV or low PIs by NT-ELISA. The overall agreement (k value) between the two tests was 0.86. A good correlation ($r^2 = 0.77$) was also observed between the tests for endpoint titration of sera ($n = 116$). In conclusion, the newly developed NT-ELISA may be a good alternative serologic assay for detecting WNV that can be used for large-scale testing of WNV-neutralizing antibodies in multiple species.

West Nile virus (WNV) infection causes encephalitis and has been recognized as one of the most widespread arboviral infections in a variety of species, including humans, birds, and horses. The geographical distribution of WNV includes Africa, the Middle East, Southern Europe, Asia, and North America (8). Recently, encephalitis epidemics caused by WNV infection have been reported in Romania (1996), Russia (1999), Israel (1999 and 2000), and North America (1999 to the present) (4, 8, 11, 16, 26, 32).

While WNV is capable of causing severe meningoencephalitis, primarily in horses, humans, and wild birds, infection in the majority of vertebrate species exposed to WNV remains subclinical or asymptomatic. In nature, wild birds play a critical role as amplifying hosts in the WNV transmission cycle, which involves primarily *Culex* mosquitoes as the transmission vector (17). Humans and horses are thought to be incidental dead-end hosts (36).

The presence of protective and neutralizing antibodies in affected animals is one of the principal factors that prevents the development of clinical disease due to WNV infection. As for other flaviviruses, the envelope (E) protein of WNV is the primary antigen and plays a critical role in the development of

protective immunity against WNV (2, 7, 10, 24, 34) by inducing the production of protective, antiviral, neutralizing antibodies. Therapeutic studies in mice demonstrated that neutralizing monoclonal antibodies (MAbs) to the E protein protected mice against WNV-induced mortality (24). Thus, it appears that the production of neutralizing antibodies to the E protein is an important aspect of the immune response to WNV infection and a goal of vaccine development as a preventive measure.

Various types of vaccines for WNV have been explored for their ability to protect susceptible hosts against pathogenic WNV infection: formalin-inactivated (18, 22), live attenuated (37), and recombinant chimeric virus vaccines (1, 10, 15, 20, 27); recombinant PrM/E or E protein vaccines (28, 34); and DNA-based vaccines (9, 12, 33). Currently, a formalin-inactivated WNV vaccine (West Nile-Innovator; Fort Dodge Animal Health, IA) and a recombinant canarypox virus vector-based vaccine expressing PrM/E proteins of WNV (Recombitek; Merial Limited, GA) are commercially available for veterinary use in the United States (23).

The plaque reduction neutralization test (PRNT) is the "gold standard" serologic assay for WNV and is currently available for measuring protective and neutralizing antibodies in serum. The test, however, takes several days to complete and requires an environment with a high level of biosafety for manipulating infectious WNV. Furthermore, the PRNT is not suitable for large-scale screening of susceptible animals, i.e.,

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for monitoring population (or herd) immunity or measuring vaccine efficacy and infection. Recently, several enzyme-linked immunosorbent assays (ELISAs) have been developed and used in serologic testing for WNV infection, mainly in humans and horses (3, 5, 35). Although these ELISAs have been useful in detecting exposed individuals, test results do not directly correlate with the development of protective immunity against WNV in those individuals. Recently, an approach for measuring antibody-mediated neutralization of WNV infection using virus-like particles that measure infection as a function of reporter gene expression was reported (25).

In this report, we describe a simple method for measuring WNV-neutralizing serum antibodies using a competitive ELISA, which utilizes a neutralizing MAb against WNV.

MATERIALS AND METHODS

Viruses and cells. WNV strains NY385-99 and B956 (American Type Culture Collection, Manassas, VA) were used. The NY385-99 strain (lineage I) of WNV was isolated from a snowy owl in New York during the 1999 epizootic (31), and the B956 strain (lineage II) was isolated from a woman in Uganda in 1937 (30). All viruses were grown in Vero cells (ATCC CCL81) in alpha minimum essential medium (GibcoBRL) supplemented with 5% fetal bovine serum (GibcoBRL) and an antibiotic-antimycotic mixture (Invitrogen, Carlsbad, CA). All virus manipulations were performed in a biosafety level (BL) 3 maximum-containment research laboratory at the National Veterinary Research and Quarantine Service in accordance with the regulations of the Korean government.

ELISA antigen. Cell culture-derived WNV was inactivated by incubation with 1% (vol/vol; final concentration, 1 mM) 0.1 M binary ethylenimine (0.205 g 2-bromoethylamine hydrobromide in 10 ml 0.2 N sodium hydroxide) for 4 h at 37°C, and the reaction was stopped by the addition of sodium thiosulfate at a final concentration of 10 mM. Inactivated virus was aggregated using 7% polyethylene glycol 8000 in the presence of 0.5 M sodium chloride overnight at 4°C, precipitated by centrifugation (5000 × g, 30 min), resuspended in 1/100 volume of 0.01 M phosphate-buffered saline (PBS [pH 7.4]), and then concentrated by dialysis. The total protein concentration of the antigen preparation was measured using a GeneQuant II (Pharmacia Biotech) according to the manufacturer's instructions and adjusted to 0.1 mg/ml. The inactivated WNV antigen was used for immunizing mice and for the ELISA.

Monoclonal antibodies. Two E protein-specific MAbs, 5E8 (neutralizing) and 2F10 (nonneutralizing), generated in our laboratory against the New York strain NY385-99 (lineage I WNV), were used in this study. Neutralizing MAb 5E8 (immunoglobulin G1 [IgG1] κ subtype) contacts at least three residues in domain III of the E protein, E330, E332, and E367, and neutralizes infection of Vero cells by both lineages of WNV (6). Nonneutralizing MAb 2F10 [IgG2b(κ) subtype] binds to recombinant E protein but does not inhibit binding of MAb 5E8 to WNV in a competitive ELISA. The two MAbs bound to WNV but not to other flaviviruses, including Japanese encephalitis virus, Saint Louis encephalitis virus, yellow fever virus, and dengue virus, on Panbio arbovirus indirect fluorescent-antibody assay slides (Panbio, Columbia, MD).

The two MAbs were purified using an ImmunoPure (recombinant protein A/G) IgG purification kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Where indicated, purified MAb 5E8 was conjugated to peroxidase using a peroxidase labeling kit (Roche, Manheim, Germany). Briefly, 0.3 ml of purified MAb (4.0 mg/ml) in sodium carbonate-hydrocarbonate buffer (100 mM, pH 9.8) was coupled to 0.1 ml activated peroxidase solution (16 mg/ml) for 2 h at 25°C. The reaction was stopped by the addition of 40 μl of 2 M triethanolamine solution (pH 9.8) for 30 min at 4°C and an additional 25 μl of the same solution for 2 h at 4°C. After the addition of 10 μl of 1 M glycine solution (pH 7.0), the MAb conjugate was allowed to dialyze extensively and was then suspended in the same volume of stabilizing reagent (enclosed in the kit). The MAb conjugate solution was stored at 4°C for short periods or was lyophilized for longer storage at 4°C.

Sera. A total of 458 equine field-collected serum samples from WNV-vaccinated ($n = 212$) and nonvaccinated ($n = 246$) horses were used. Equine sera consisted of sera from horses ($n = 212$) vaccinated with a formalin-inactivated WNV vaccine prior to export from the United States to South Korea and sera from Mongolian horses ($n = 98$) and Korean horses ($n = 148$) that had not received a flaviviral vaccine; the sera were kindly provided by R. Sodnomdarjaa

TABLE 1. NT-ELISA of sera from WNV-infected mice

WNV strain	No. of mice	NT-ELISA results[a]		
		0 dpi	7 dpi	14 dpi
NY385-99 (lineage I)	12	7.0 ± 7.6	95.2 ± 1.4	93.4 ± 2.1
B956 (lineage II)	9	-5.7 ± 7.5	89.9 ± 1.5	ND[b]

[a] Values represent mean PIs ± standard deviations. Sera taken at 7 and 14 dpi had neutralizing antibody levels based on $PRNT_{90}$ values of ≥100 for WNV, while neutralizing antibodies were undetectable in mock-infected sera by PRNT. At 0, 7, and 14 dpi, 5, 4, and 3 animals, respectively, were sacrificed for testing.
[b] ND, not done.

(State Central Veterinary Laboratory, Mongolia) and Soo-Gil Lee (Korean Racing Association, Republic of Korea), respectively.

A total of 21 sera from 6-week-old BALB/c mice were used. Mice were inoculated intraperitoneally with 10^3 PFU of WNV strain NY385-99 or B956 and sacrificed for serum collection 0, 7, and 14 days postinoculation (dpi) (see Table 1).

To prepare positive-control sera specific for WNV, two rabbits that had been experimentally infected with WNV strain NY385-99 were subsequently injected with 10^7 PFU of homologous WNV 3 weeks after the first inoculation. The rabbits were bled 2 weeks after the booster inoculation. Pooled sera from mock-infected rabbits ($n = 2$) were used as the negative control. Anti-WNV (NY385-99) hyperimmune rabbit serum had a $PRNT_{90}$ titer (expressed as the reciprocal of the highest dilution yielding ≥90% reduction in the number of plaques) of approximately 5,000 against WNV and was diluted 1:5 and 1:50 in rabbit negative-control serum to generate strongly positive and weakly positive control sera, respectively. All infected animals were kept in a BL 3 maximum-containment research laboratory at the National Veterinary Research and Quarantine Service in accordance with the regulations of the Korean government.

NT-ELISA. For the ELISA using neutralizing MAb 5E8 (NT-ELISA), Maxisorp ELISA plates were coated with MAb 2F10 (2.5 μg/ml) in 0.01 M PBS (pH 7.4), 50 μl/well, and then incubated for 1 h at 37°C. The plates were washed three times with 0.01 M PBS containing 0.05% Tween 20 and then incubated with 50 μl of the inactivated WNV antigen (total protein of 0.1 mg/ml) described above in blocking buffer (0.01 M PBS containing 0.05% Tween 20 and 5% skim milk) for 1 h at 37°C. After being washed three times, the plates were incubated with 50 μl of a mixture containing an equal volume of peroxidase-conjugated MAb 5E8 and serial dilutions of test sera for 1 h at 37°C. Strongly positive, weakly positive, and negative-control sera were included. After being washed, the plates were incubated for 10 min with the substrate O-phenylenediamine (Sigma-Aldrich) in 0.05 M phosphate-citrate buffer (pH 5.0) containing 0.015% hydrogen peroxide (OPD solution). The colorimetric reaction was stopped by adding 50 μl of 1.25 M sulfuric acid to each well. The optical density (OD) was measured at a wavelength of 492 nm. The OD of each well was converted to percent inhibition (PI) of MAb binding by competition with serum antibodies using the following formula: PI = [1 − (OD of serum-MAb mixture/OD of MAb alone)] × 100.

PRNT. The PRNT was carried out in 24-well tissue culture plates according to the procedure of the USDA National Veterinary Services Laboratories in Ames, IA, with some modifications. Briefly, 100 μl of the serum to be tested was heat inactivated and mixed with an equal volume of WNV strain NY385-99 at a concentration of 100 PFU in the presence of 10% (vol/vol) guinea pig complement (Biotechnics Research, Inc., CA) and then incubated at 37°C for 75 min. Serum samples were diluted 1:10 and 1:100 for screening or serially diluted by a twofold dilution technique for endpoint titration. Each of the serum-virus mixtures was added to a confluent monolayer of Vero cells in 24-well tissue culture plates. After adsorption for 1 h at 37°C, the cells were overlaid with overlay medium (Earle's basic salts solution containing 1% Noble agar, 2% fetal bovine serum, and antibiotics) and further incubated at 37°C for 3 days while cytopathic effects were monitored. After 3 days, a second overlay medium containing 0.006% neutral red was added to each well. After an additional 18 to 24 h, the number of plaques in each well was counted. PRNT titers ($PRNT_{90}$ values) were expressed as the reciprocal of the highest dilution yielding a ≥90% reduction in the number of plaques. Serum samples having a PRNT titer of ≥1:10 were considered to be antibody positive.

Statistical analysis. The correlation between NT-ELISA and PRNT results was evaluated by a linear regression method and expressed as the correlation coefficient (r). The agreement between both tests was calculated by the kappa quotient (19).
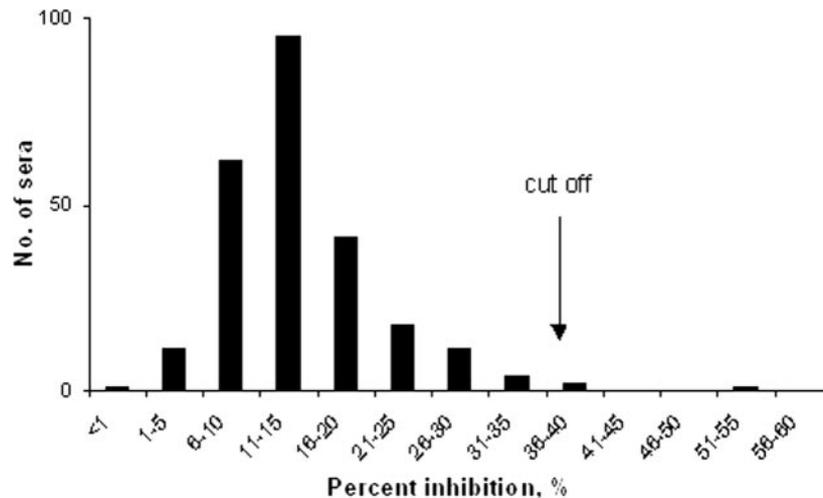
FIG. 1. Distribution of PI values for 246 naïve sera from horses negative for WNV.

RESULTS

Development of the NT-ELISA. The design of the NT-ELISA was based on competitive binding of neutralizing MAb 5E8 and serum WNV antibodies from infected animals. To minimize nonspecific binding due to cellular proteins or the other factors, nonneutralizing MAb 2F10 was used to capture WNV E protein for the NT-ELISA.

The amounts of each reagent used in the NT-ELISA were optimized using a checkerboard titration. The optimal dilution of WNV antigen (1:200) was selected based on the absorbance at 492 nm showing saturation of plates coated with MAb 2F10 (2.5 μg/ml). The optimal dilution of peroxidase-conjugated MAb 5E8 was 1:500, which had an optical density of 1.14 ± 0.05 corresponding to 75% maximum absorbance at 492 nm. The 1:10 dilutions of control rabbit sera (strongly and weakly positive control sera and mock-infected negative-control sera) and the 1:500 dilution of peroxidase-conjugated MAb 5E8 could optimally differentiate positive sera from normal negative sera.

Using the conditions just described, mean PI values for strongly positive (1:5 dilution in negative serum), weakly positive (1:50 dilution in negative serum), and negative rabbit sera were 95%, 60%, and 5.2%, respectively. For a total of 246 equine sera from nonvaccinated horses negative for WNV by PRNT, the mean PI value and standard deviation were 14.0% and 6.9, respectively (Fig. 1). Hence, the cutoff PI value was set at 35%, which is approximately equal to the mean PI value of the negative sera plus 3 standard deviations.

Evaluation of the NT-ELISA for the detection of WNV antibodies. Analysis of the performance of the NT-ELISA in the detection of WNV-neutralizing antibodies in experimentally infected mice is summarized in Table 1. In mice, the NT-ELISA detected WNV antibodies at 7 dpi in WNV strain NY385-99-infected animals (PI value of 95.2 ± 1.4) and in WNV strain B956-infected animals (PI value of 89.9 ± 1.5). The NT-ELISA was also able to detect WNV antibodies in sera from NY385-99-infected mice at 14 dpi (PI value of 93.4 ± 2.1). All of the sera collected from experimentally infected mice at 7 and 14 dpi that were seropositive for WNV-neutralizing antibodies had a $PRNT_{90}$ titer of ≥100 for WNV. In contrast, sera collected at 0 dpi from mice ($n = 10$) were negative for WNV antibodies by both NT-ELISA (PI values of <35) and PRNT ($PRNT_{90}$ titer of <10).

Correlation between NT-ELISA and PRNT. The performances of the serologic NT-ELISA and the PRNT using sera from vaccinated horses are compared in Table 2. Approximately 85% (180/212) of the samples from vaccinated horses and none of the samples from nonvaccinated horses were positive by PRNT using a $PRNT_{90}$ titer of 10 as the cutoff. In contrast, the NT-ELISA yielded a positive result for 96.1% (173/180) of the PRNT-positive sera and 3.1% (1/32) of the PRNT-negative sera. Sera ($n = 7$) that were negative by NT-ELISA had a low level of neutralizing antibody ($PRNT_{90}$ titers of ≤100). One serum sample that was negative by PRNT had a low PI by NT-ELISA (PI = 46%). The overall agreement (k value) between the results of the two tests was 0.86.

An endpoint titration using the NT-ELISA was performed using serial dilutions of PRNT-positive sera ($n = 116$) to analyze whether the NT-ELISA titers correlated with PRNT titers for WNV. PI values determined using the NT-ELISA showed a good correlation ($r^2 = 0.77$) with $PRNT_{90}$ titers (Fig. 2), although overall antibody titers obtained by NT-ELISA were on average 1.32 log 2 lower than those obtained by PRNT.

TABLE 2. NT-ELISA of field-collected sera from WNV-vaccinated horses[a]

NT-ELISA results[b]	PRNT results[c]	
	Positive ($n = 180$)	Negative ($n = 32$)
Positive ($n = 174$)	173	1
Negative ($n = 38$)	7	31

[a] Agreement (k) between the two tests was 0.86.
[b] Sera having PI values of >35 at a final dilution of 1:10 were considered positive by NT-ELISA. All sera were tested in duplicate.
[c] Results are given in numbers of serum samples. Sera having $PRNT_{90}$ titers of >10 were considered positive by PRNT for WNV.
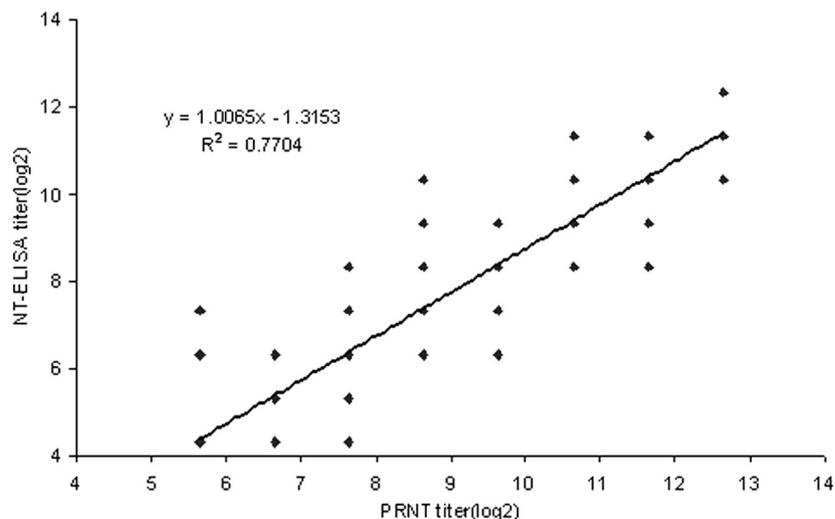
FIG. 2. Correlation between PRNT and NT-ELISA results for antibody titration in 116 sera from WNV-vaccinated horses. One point may represent several sera with the same result. Reciprocals of the highest dilution of sera having PI values of >35 were determined by NT-ELISA.

DISCUSSION

The PRNT, based on cell culture methodology, has been used extensively for the detection and titration of neutralizing antibodies in flaviviral infections. The PRNT, however, has limitations, in that it is labor-intensive and time-consuming and requires biosecurity facilities capable of protecting humans from exposure to infectious viruses. As a result, detection of neutralizing antibodies induced by natural infection or vaccination has been hampered by the lack of easy and specific tests. Compared to the PRNT, the NT-ELISA developed in this study provides simplicity, a short turn-around time, and biohazard safety for the detection of neutralizing antibodies. The NT-ELISA is the preferred method for serologic screening of numerous serum samples for WNV, such as would be required for seroepidemiological surveys and vaccine efficacy studies.

The 5E8 antibody used to develop the NT-ELISA in this study recognizes a neutralizing epitope in domain III of the E protein of WNV. Neutralizing antibodies induced by domain III of flaviviral E proteins have been reported elsewhere as a major factor responsible for the greatest protection in vivo (24, 29), and all types of recently developed bioengineered vaccines target the E protein (1, 9, 10, 12, 15, 20, 27, 28, 33, 35). In our assay, competition for binding of MAb 5E8 by serum antibodies was observed in most of the serum samples from vaccinated horses (96.1%) as well as in serum samples from early infected mice. These results indicated that the epitope recognized by the 5E8 antibody is immunodominant and is a suitable target for the detection of neutralizing antibodies in sera.

There are several examples of the use of recombinant E proteins of WNV to develop diagnostic tests that are based on the indirect ELISA format (3, 13, 21, 35). However, in a preliminary study using a competitive ELISA format, we found that WNV serum-neutralizing antibodies in most equine sera did not inhibit 5E8 binding to recombinant E protein expressed in *Escherichia coli*, perhaps due to conformational alterations in critical antigenic structures in the recombinant E

protein. For this reason, we used binary ethylenimine-inactivated whole-virus antigen for the NT-ELISA.

Such recombinant proteins, if evaluated fully by NT-ELISA, may have advantages over inactivated whole-virus antigen, in that recombinant proteins are produced in a safe manner without the need for a BL 3 containment facility. Therefore, in order to replace the whole-virus antigen in the NT-ELISA, we are investigating the efficacy of recombinant proteins with correct antigenic structures, generated using native whole-virus particles such as recombinant virus-like particles (14, 25, 28).

The novel NT-ELISA demonstrated in the current study showed good relative sensitivity (96.1% versus PRNT) in vaccinated horses and correlated well with the PRNT in endpoint titration ($r^2 = 0.77$), although differences between the two tests were observed in some of the serum samples that had a weak level of neutralizing antibodies ($PRNT_{90}$ titers of between 1:10 and 1:100). This indicates that the NT-ELISA may be useful for rapidly examining the status of the protective immune response in vaccinated individuals or herds of interest (i.e., horses), while the protective level of neutralizing antibodies may require further analysis. For example, horse populations that were previously vaccinated could be screened rapidly by NT-ELISA to detect poorly immunized individuals.

Failure of the NT-ELISA to detect antibodies in some of the weakly positive sera might be explained by the observation that serum titers determined by ELISA were generally 2.5-fold lower than $PRNT_{90}$ titers in endpoint titration, indicating that some of the weakly positive sera, as assessed by PRNT, managed to become negative in the NT-ELISA. To account for the lower sensitivity, a lower cutoff (i.e., the mean PI value of negative equine sera plus 2 standard deviations) or a lower serum dilution of <1:10 might be used.

Finally, our results with experimentally infected mice and horses revealed that our NT-ELISA could also be applied to serologic surveillance and diagnosis of West Nile encephalitis in a variety of susceptible animal species, if fully evaluated,

similar to the epitope-blocking ELISA developed for WNV serology in multiple avian species (5).

ACKNOWLEDGMENTS

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